

## RESEARCH PAPER

# Inhibition of CXCR3-mediated chemotaxis by the human chemokine receptor-like protein CCX-CKR

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## BACKGROUND AND PURPOSE

Induction of cellular migration is the primary effect of chemokine receptor activation. However, several chemokine receptor-like proteins bind chemokines without subsequent induction of intracellular signalling and chemotaxis. It has been suggested that they act as chemokine scavengers, which may control local chemokine levels and contribute to the function of chemokines during inflammation. This has been verified for the chemokine-like receptor proteins D6 and DARC as well as CCX-CKR. Here, we provide evidence for an additional biological function of human (h)CCX-CKR.

## EXPERIMENTAL APPROACH

We used transfection strategies in HEK293 and human T cells.

## KEY RESULTS

Co-expression of hCCX-CKR completely inhibits hCXCR3-induced chemotaxis. We found that hCCX-CKR forms complexes with hCXCR3, suggesting a relationship between CCX-CKR heteromerization and inhibition of chemotaxis. Moreover, negative binding cooperativity induced by ligands both for hCXCR3 and hCCX-CKR was observed in cells expressing both receptors. This negative cooperativity may also explain the hCCX-CKR-induced inhibition of chemotaxis.

## CONCLUSIONS AND IMPLICATIONS

These findings suggest that hCCX-CKR prevents hCXCR3-induced chemotaxis by heteromerization thus representing a novel mechanism of regulation of immune cell migration.

## Abbreviations

FCS, fetal calf serum; Pen/Strep, penicillin/streptomycin; PHA, phytohemagglutinin

## Introduction

Chemokines, small proteins that regulate cell migration, play important roles in the immune system, angiogenesis, hematopoiesis, cell differentiation and development (Rossi and Zlotnik, 2000; Horuk, 2001). Chemokines and their receptors have been classified into four different subgroups based on the presence and position of conserved cysteine residues i.e. CXC, CC, C and CX3C (Murphy *et al.*, 2000). Chemokine receptors are GPCRs that signal via G-proteins to various signalling pathways (Sanchez-Madrid and del Pozo, 1999; Thelen, 2001). In addition, atypical chemokine receptors represent a subfamily of chemokine-binding proteins that do not signal along classic GPCR-mediated pathways, but efficiently internalize their cognate ligands and act as chemokine scavengers (Hansell *et al.*, 2006). In addition to DARC and D6, CCX-CKR belongs to this family of proteins and scavenges its ligands CCL21, CCL19, CCL25 and CXCL13 (Gosling *et al.*, 2000; Townson and Nibbs, 2002; Comerford *et al.*, 2006). CCX-CKR is widely expressed in several tissues, including the brain and is expressed in many immune cells such as microglia, dendritic cells and T cells together with other chemokine receptors (Gosling *et al.*, 2000; Murphy, 2002; Townson and Nibbs, 2002; Zuurman *et al.*, 2003; Brouwer *et al.*, 2004). It is well known that chemokine receptors like other GPCRs can, when co-expressed, form heteromers that exhibit altered intracellular signalling properties (Rodriguez-Frade *et al.*, 1999; Rios *et al.*, 2001; Springael *et al.*, 2005; 2006). Because CCX-CKR is found co-expressed with other chemokine receptors, we investigated whether co-expression of CCX-CKR might change the signalling of other chemokine receptors in addition to its function as scavenger.

## Methods

### Chemicals

Media, sera, and reagents used for cell culture were purchased from PAA Laboratories (Colbe, Germany). All other chemicals were from Sigma-Aldrich (Zwijndrecht, The Netherlands), unless mentioned otherwise.

### Cell cultures

HEK293 cells and HEK293T (gift from Dr S. Carra, Department of Cell Biology, UMCG, Groningen, Netherlands) were cultured in growth medium [DMEM, supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% Pen/Strep (Invitrogen, Breda, The Netherlands) and 1% sodium pyruvate] and kept in a humidified atmosphere (5% CO<sub>2</sub>) at 37°C.

### T cell isolation and activation

Peripheral blood mononuclear cells were isolated from heparinized blood taken from healthy donors using a Ficoll (GE Healthcare, Diegem, Belgium) gradient centrifugation for 15 min at 1000 g without brake. T cells were isolated by co-incubating the mononuclear cells with 2-(2-aminoethyl) isothioureia dihydrobromide (AET)-treated sheep red blood cells (Oxiod, Badhoevedorp, The Netherlands) for 15 min on ice. Erythrocyte rosettes were separated from the mononu-

clear cells by Ficoll gradient centrifugation for 15 min at 1000 g without brake. Subsequently, the sheep red blood cells were lysed using Gey's lysis buffer (7.0 g·L<sup>-1</sup> NH<sub>4</sub>Cl, 0.37 g·L<sup>-1</sup> KCl, 0.3 g·L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, 0.024 g·L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 1.0 g·L<sup>-1</sup> glucose, 10.0 mg·L<sup>-1</sup> phenol red, 8.4 mg·L<sup>-1</sup> MgCl<sub>2</sub>·6H<sub>2</sub>O, 7.0 mg·L<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, 6.8 mg·L<sup>-1</sup> CaCl<sub>2</sub> and 45 mg·L<sup>-1</sup> NaHCO<sub>3</sub>), yielding a population of 92.5 ± 4.6% CD3<sup>+</sup> T cells. Freshly isolated T cells were used directly for further experiments or resuspended in growth medium [RPMI 1640 (Invitrogen), supplemented with 5% FCS (Invitrogen), 100 U·mL<sup>-1</sup> penicillin, 100 µg·mL<sup>-1</sup> streptomycin] and kept in a humidified atmosphere (5% CO<sub>2</sub>) at 37°C. T cells were activated by stimulating them with 2 µg·mL<sup>-1</sup> phytohemagglutinin (PHA; Biotrading, Mijdrecht, The Netherlands) for 24 h and with 25 mL<sup>-1</sup> IL-2 (PeproTech, London, UK) for another 3–5 days.

### Plasmids

The following constructs were used for generating stable cell lines: pcDNA3.1 plasmid containing the full-length sequence of human CXCR3 [hCXCR3; kind gift of C. Tensen and B. Moser (Hensbergen *et al.*, 2001)]; pcDNA3.1 containing full-length human CCX-CKR [hCCXCKR; kindly provided by R. Nibbs (Townson and Nibbs, 2002)]; pREP9 containing full-length CX3CR1 [kindly provided by P.M. Murphy (Combadiere *et al.*, 1995)].

The following constructs were used for FRET experiments: hCXCR3, hCCX-CKR, GABAB-R1 and hCCR5 coupled both to Venus or Cyan Fluorescent Protein (CFP). pcDNA3.1 (+)-GABAB-R1 [kindly provided by B. van Lith (Molecular Pharmacology & DMPK, Schering-Plough Research Institute, Oss, The Netherlands)] was used as negative control. hCCR5 [Missouri S&T cDNA Resource Center (<http://www.cdna.org>)] served also as negative control. All four constructs were subcloned using primers described in Table 1. hCXCR3 and hCCX-CKR were inserted into pECFP-N1 and pVenus-N1 vector using EcoRI and SmaI as restriction enzymes. hCCR5 was inserted using HindIII and SmaI while hGABAB-R1 was inserted using EcoRI and SmaI. hGABAB-R1 subcloning was done in presence of 10% glycerol. The pVenus-N1 vector was generated using the pECFP-N1 (Clontech) as backbone. ECFP was cut out of the pECFP-N1 plasmid using AgeI and NotI. Venus was cut out of the pcDNA3.1 CFP-EPAC-Venus construct using XhoI. Venus was subcloned (see primers in Table 1) and inserted into the pECFP-N1 (after deletion of ECFP) using AgeI and NotI.

The following constructs were used for transient transfection followed by chemotaxis assays to verify whether hCCX-CKR is affecting other chemokine receptors: hCCR2, hCCR4, hCCR5, hCCR6, hCCR7, hCCR9, hCCR10 and hCXCR4. These constructs have been purchased from: Missouri S&T cDNA Resource Center (<http://www.cdna.org>).

### Generation of stable HEK293 cell lines

Cells were transfected with either hCXCR3, hCCX-CKR or both constructs using FugeneR (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. Stable polyclonal transfected cells were selected using 500 µg·mL<sup>-1</sup> G-418 (Omnilabo, Breda, The Netherlands) for approximately 2 weeks. Hereafter, monoclonal cell lines were generated and maintained for maximum 15 passages.

**Table 1**

Primers used for cloning for FRET constructs

Gene of interest	Forward primer	Reverse primer
CXCR3	AAAGAATTCTAATGGTCCTTGAGGTGAGTGACC	AAACCCGGGCCAAGCCCGAGTAGGAGGCCTC
CCXCKR	AAAGAATTCTAATGGCTTTGGAACAGAACAGTC	AAACCCGGGCAATGCTAAAAGTACTGGTTGGCTCT
GABAB-R1	AAAGAATTCTGATGTTGCTGCTGCTGTTACTGGCG	AAAAGTCGACTGCTTATAAAGCAAATGCACTCGACTCC
CCR5	AAAAAAGCTTATGGATTATCAAGTGCAAGTCCAAT	AAACCCGGGCCAAGCCACAGATATTTCTGCTC
Venus	AAAACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCT	AAAGCGCCGCTTACTTGTACAGCTCGTCCATGCCGA

### Cell transfection

HEK293 and HEK293T cells were transfected using calcium/phosphate method. Briefly, cells were kept in DMEM without additives and were left with DNA precipitates for 5 h. After transfection, cells were rinsed with DMEM and put back in growth medium. Mock transfections were done using empty plasmids.

For radioligand-binding experiments, HEK293T cells were transiently transfected with 1 µg receptor-encoding plasmid DNA using 25-kDa linear polyethyleneimine (Polysciences, Eppenheim, Germany) as described previously (Verzijl *et al.*, 2008). The total amount of transfected DNA was adjusted to 5 µg with the empty vector pcDEF3.

hCCX-CKR was silenced in freshly isolated T cells using pLKO.1-puro vectors containing DNA encoding for CCX-CKR short-hairpin silencing RNA (shRNA) (MISSION® shRNA NM\_16557; Sigma-Aldrich). Five vectors for hCCX-CKR silencing were used for each different T cell donor. Plasmid containing GFP-encoding DNA (pmaxGFP; Lonza, Basel, Switzerland) was used as positive control for transfection and the pLKO.1-puro vector containing DNA encoding for non-targeting shRNA (MISSION® shRNA SHC002; Sigma-Aldrich) was used as negative control. T cells were transfected using the Amaxa nucleofactor kit for human T cells (Lonza) according to manufacturer's instructions. Briefly,  $2 \times 10^6$  to  $5 \times 10^6$  T cells were resuspended in specific nucleofactor solution and mixed with 2 µg vector DNA. Subsequently, the cells were nucleofected using the T cell-specific programme V-24 and transferred into growth medium. hCCX-CKR was overexpressed in PHA/IL-2-treated T cells using the same method. T cells were resuspended in specific nucleofactor solution, mixed with 5 µg of hCCX-CKR plasmid DNA or pmaxGFP, and nucleofected by using the T cell specific programme T-20. Transfection efficiency and hCCX-CKR expression were assessed by flow cytometry 24 h (overexpression) or 48 h (silencing) post-nucleofection.

### Flow cytometry

Expression of surface molecules by human T cells was determined using flow cytometry. Cells were incubated with pre-determined optimal dilutions of primary antibody diluted in PBS containing 0.5% BSA and 0.01% sodium azide for 10 min at RT. Primary antibodies were phycoerythrin (PE)-conjugated anti-CXCR3 (clone 49801; R&D Systems, Abingdon, UK), FITC-conjugated anti-CD3 (clone UCHT1; eBioscience, Vienna, Austria), PE-Cy5-conjugated anti-CD4 (clone RPA-T4; eBioscience), allophycocyanin (APC)-

conjugated anti-CD8 (clone RPA-T8; eBioscience), APC-conjugated anti-CD44 (clone IM-7; eBioscience), PE-Cy5-conjugated anti-CD62L (clone DREG-56; eBioscience) and polyclonal goat-anti-CCX-CKR (CCR11; Capralogics, Hardwick, MA, USA). hCCX-CKR was detected using PE-conjugated anti-goat IgG (Jackson ImmunoResearch, Newmarket, UK) as a secondary antibody. Isotype-matched primary antibodies of irrelevant specificity served as negative controls. 20,000–30,000 events were measured using a FAC-SCalibur flow cytometer (BD Biosciences, Breda, The Netherlands) and analysed by WinMDI software.

### Chemotaxis assay

Cell migration in response to chemokines was assessed using a 48-well chemotaxis microchamber (NeuroProbe, Gaithersburg, MD, USA). 10 µM chemokine stock solutions (containing recombinant human CCL4, CCL13, CCL17, CCL20, CCL21, CCL25, CCL27, CXCL9, CXCL10 or CXCL12; R&D Systems) were prepared in sterile PBS and further diluted in serum-free DMEM for use in the assay. Serum-free DMEM without chemokines served as control. 27 µL of the chemoattractant solution or control medium was applied to the lower well of the chamber. Upper and lower chamber were separated by a polyvinylpyrrolidone-free polycarbonate filter (5-µm pore size for T cells and 8-µm pore size for HEK293/HEK293T cells). In the upper wells of the chamber, 50-µL cell suspension containing  $5 \times 10^4$  cells was applied. The chamber was incubated in a humidified atmosphere (5% CO<sub>2</sub>) at 37°C for 2 h. Determinations were done in hexaplicate for each group. After incubation the filter was washed, fixed in methanol and stained with toluidine blue. Migrated cells per group were counted by a double-blinded person with a scored eyepiece [three fields (1 mm<sup>2</sup>) per well].

### Reverse transcription and quantitative real-time PCR (QPCR)

T cells were lysed in guanidinium isothiocyanate/mercaptoethanol buffer and total RNA was extracted according to Chomczynski and Sacchi (1987) with slight modifications. One µg of total RNA was transcribed into cDNA as described (Biber *et al.*, 1997). Primers used for RT-PCR are described in the Supporting Information Table S1. Real-time PCR, using an iCyclerR (Bio-Rad, Veenendaal, The Netherlands) and iQ SYBR Green supermixR (Bio-Rad), was performed on 4 ng cDNA from T cells. Primers (Supporting Information Table S1) for QPCR, yielding PCR products of approximately 100 base pairs long, were designed by 'Primer Designer'

(Version 3.0, Scientific and Educational Software, Cary, NC, USA). PCR reactions with primers for hCCX-CKR and hCXCR3 were run in parallel with primers for the housekeeping genes GAPDH and hypoxanthine guanine phosphoribosyl transferase. All primers were purchased from Biolegio (Nijmegen, The Netherlands). Melt-curve analysis was performed immediately following amplification in order to check primer specificity. For analysis, the comparative Ct method was used. In each experiment samples were run in duplicate. Results are the averaged data of three independent experiments and are given as mean  $\pm$  SEM.

### Immunocytochemistry

HEK293 cells were plated on poly-L-lysine (PLL) coated glass coverslips and fixed in 4% paraformaldehyde (PFA) solution. Cells were pre-incubated in PBS containing 10% FCS and 0.3% Triton-X100 for 30 min at room temperature. Incubation with primary antibodies against hCXCR3 (R&D, 1:100) and hCCX-CKR (Capralogics, 1:400) was performed O/N at 4°C. The next day, cells were incubated with secondary antibodies conjugated with fluorescent labels (Jackson Immuno Research) for 2 h at room temperature. Cells were washed, stained with Hoechst nuclear dye and finally mounted in Vectashield Mounting Media (Vector, Peterborough, UK). Stained cells were analysed with a Zeiss Axioskop 2 microscope and confocal fluorescence microscopy (see FRET section).

### Ligand-binding experiments

The CXCR3 antagonist VUF10085 was first described in literature as AMG 487 (Johnson *et al.*, 2007) and re-synthesized in our department. [<sup>125</sup>I]-CXCL10 and Na<sup>125</sup>I were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). CCL19 was purchased from Peprotech (Rocky Hill, NJ, USA) and labelled with Na<sup>125</sup>I using iodogen pre-coated tubes (Thermo Fischer Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Twenty-four hours post-transfection, HEK293T cells were transferred to poly-L-lysine (Sigma-Aldrich) coated 48-well plates. The next day, cells were incubated for 4 h at 4°C with 0.5–1 nM [<sup>125</sup>I]-CCL19 or 50–100 pM [<sup>125</sup>I]-CXCL10 in binding buffer (50 mM HEPES, 100 mM NaCl, 1 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, pH 7.4, 0.5% BSA) containing unlabeled displacer. After incubation, cells were washed three times with ice-cold wash buffer (50 mM HEPES, 0.5 M NaCl, 1 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, pH 7.4) and subsequently lysed. Cell lysates were counted in a Wallac Compu-gamma counter (PerkinElmer Life and Analytical Sciences). For ligand binding cooperativity assays, percentages of CXCR3-specific [<sup>125</sup>I]-CXCL10, [<sup>125</sup>I]-CXCL11 and CCX-CKR-specific [<sup>125</sup>I]-CCL19 binding were calculated by normalizing data to binding in the absence of unlabeled displacer (100%) and binding in the presence of 10 μM VUF10085, 100 nM CXCL11 and 100 nM CCL19, respectively (0%). For homologous radioligand displacement curves, CXCR3-specific [<sup>125</sup>I]-CXCL10, [<sup>125</sup>I]-CXCL11 and CCX-CKR-specific [<sup>125</sup>I]-CCL19 binding were calculated by normalizing data to radioligand binding in the absence of unlabeled displacer (100%) and in the presence of 10 μM VUF10085, 100 nM CXCL11 and 100 nM CCL19, respectively (0%), in cells expressing only CXCR3 ([<sup>125</sup>I]-CXCL10 and [<sup>125</sup>I]-CXCL11) or only CCX-CKR ([<sup>125</sup>I]-CCL19).

### ELISA measurements

Cells were plated in 48-well plates, fixed 30 min with 4% PFA in PBS 48 h after plating and washed two times with TBS. For the permeabilized samples, cells were incubated 30 min with 0.5% NP-40 (Roche Molecular Biochemicals) in TBS after fixation. Samples were blocked for 4 h at RT with 1% fat-free milk in 0.1 M NaHCO pH 8.6. Primary antibody (R&D Systems, MAB160) was diluted 1:1000 in 0.1% BSA in TBS and incubated O/N at 4°C and washed three times with TBS. Secondary antibody (Bio-rad 170–6516 goat-anti-mouse/HRP) was diluted 1:2500 in 1% fat free milk in 0.1 M NaHCO pH 8.6 and incubated 3 h at RT. o-Phenylenediamine substrate solution [2.2 mM ophenylenediamine (Sigma-Aldrich P-1526), 35 mM citric acid, 66 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.015% H<sub>2</sub>O<sub>2</sub>, pH 5.6) was added. The reaction was stopped by addition of 1 M H<sub>2</sub>SO<sub>4</sub> and the absorbance at 490 nm was measured with a Wallac Victor plate reader.

### FRET experiments

HEK 293T cells were transfected with plasmids encoding Venus- or CFP-tagged versions of hCXCR3, hCCX-CKR or both. For control experiments Venus-tagged versions of hGABAB-R1 and hCCR5 were used. Imaging of the expression of the various fusion proteins was performed on a Leica AOBIS\_TCS SP2 confocal laser scanning microscope using an 63x NA 1.4 oil-immersion objective (Leica Microsystems) and the 458 nm line of an AR/Kr laser. The microscope was set-up and corrections were applied according to Van Rheenen *et al.* (2004). FRET efficiency was determined using the method by Jalink and Van Rheenen (2009). Shortly, images were corrected by subtracting the background and correcting for bleedthrough by using CFP- and Venus-transfected cells followed by correction of intensity. Values were measured by scaling all samples to the same level of the hCXCR3-CFP/hCXCR3-Venus homomer followed by measuring the intensity at different region of interests at the membrane.

## Results

### CCX-CKR co-expression inhibits chemotactic signalling of various chemokine receptors

In order to investigate if co-expression of hCCX-CKR influences the signalling of other chemokine receptors that don't share ligands with CCX-CKR, chemotaxis experiments were performed. We observed that the migration of HEK293 cells transfected with the chemokine receptors hCCR6, hCCR10, hCXCR3 or hCXCR4 was significantly reduced when hCCX-CKR was co-expressed (Table 2). Co-expression together with hCCR2 and hCCR4 showed weak, non-consistent reduction in the chemotactic response. Chemotaxis towards receptor hCCR5 was not inhibited by the presence of hCCX-CKR. Finally, hCCR7 and hCCR9 were also investigated, which both share common ligands with CCX-CKR. The chemotactic response of these receptors was also blunted when co-expressed with CCX-CKR (Table 2). In order to analyse the effects of co-expression in more detail, we generated HEK293 cells stably co-expressing CCX-CKR and CXCR3, an important receptor for immune cell trafficking.



**Table 2**

Chemokine receptors tested for chemotaxis inhibition by hCCX-CKR

Receptor	Ligand (1 nM)	% of migration (control is set to 100%)	n	P-value
CCR2	CCL13	125 ± 13	3	0.069
CCR2+CCX-CKR		92 ± 6		
CCR4	CCL17	139 ± 5	3	0.069
CCR4+CCX-CKR		111 ± 11		
CCR5	CCL4	143 ± 13	3	0.15
CCR5+CCX-CKR		121 ± 6		
CCR6	CCL20	135 ± 10	3	0.02
CCR6+CCX-CKR		96 ± 9		
CCR7	CCL21	125 ± 15	3	0.023
CCR7+CCX-CKR		75 ± 9		
CCR9	CCL25	168 ± 15	3	<0.001
CCR9+CCX-CKR		94 ± 5		
CCR10	CCL27	158 ± 15	3	0.003
CCR10+CCX-CKR		84 ± 11		
CXCR3	CXCL10	148 ± 9	3	<0.001
CXCR3+CCX-CKR		82 ± 7		
CXCR4	CXCL12	154 ± 16	3	0.009
CXCR4+CCX-CKR		83 ± 10		

HEK293T cells were either transfected with a chemokine receptor alone or with a combination of a chemokine receptor and CCX-CKR. Cells were then subjected to a chemotaxis experiment using the chemokine specific for the transfected receptor diluted in medium at a concentration of 1 nM. Control migration was done in presence of medium alone. In all control experiments, between 25 and 45 cells migrated towards the medium. Using these numbers, control migration was set to 100%. Data represent the mean and SEM of 3 independent experiments. ANOVA was performed to illustrate statistical differences in migration.  $P < 0.05$  indicates that the chemotaxis mediated by the chemokine receptor towards its ligand was inhibited by the presence of CCX-CKR.

### Expression of hCCX-CKR does not affect expression of CXCR3

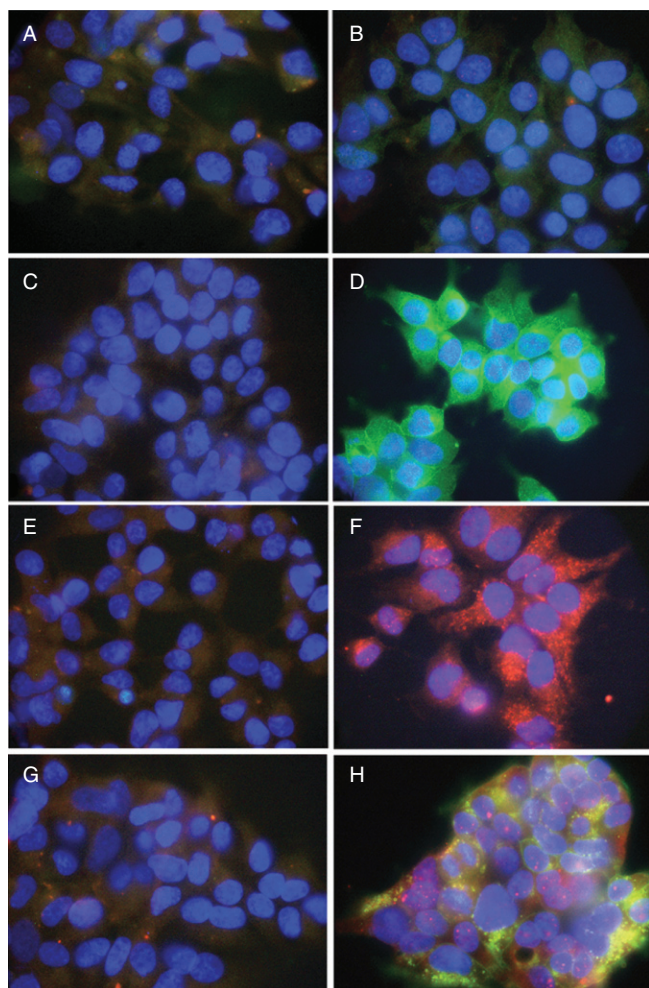
First the presence of chemokine receptors expression was examined by immunocytochemistry. Non-transfected HEK293 cells did not show staining for hCXCR3 (green signal) (Figure 1A) and hCCX-CKR (red signal) (Figure 1B). Staining for hCXCR3 was observed in both hCXCR3-transfected (Figure 1D) and double transfected HEK293 cells (Figure 1H) and hCCX-CKR expression was observed in hCCX-CKR-transfected (Figure 1F) and double-transfected HEK293 cells (Figure 1H). Omission of primary antibodies generated no signal (Figure 1C,E,G). These findings were also confirmed at mRNA level (Supporting Information Figure S1; Supporting Information Table S2). To exclude the possibility that the presence of hCCX-CKR might hamper the normal expression of hCXCR3, expression levels of hCXCR3 in these cells were further investigated by ELISA measurements (Figure 1I). We show that comparable levels of hCXCR3 expression were found in hCXCR3-transfected cells and hCXCR3 + hCCX-CKR-transfected cells. Although there is a decrease in hCXCR3 expression in presence of hCCX-CKR, which is probably due to the cytomegalovirus promoter driving the protein machinery to its limit, it is not sufficient to explain the complete inhibition of chemotaxis towards CXCL10 that we observed. Thus the co-expression of hCCX-CKR did not affect protein expression of hCXCR3 in HEK293 cells.

### Effect of CCX-CKR on CXCR3-mediated chemotaxis

Having confirmed the expression levels of our stable cell lines, we tested the effect of hCCX-CKR expression on hCXCR3-mediated migration. Whereas HEK293 cells expressing hCXCR3 cells displayed typical chemotaxis responses to CXCL9 and CXCL10, HEK293 cells expressing both hCXCR3 and hCCX-CKR failed to migrate in response to these two chemokines at all concentrations tested (Figure 2A,B). The lack of migration in double-transfected cells could have been due to an artefact as a result of the transfection procedure. To exclude this possibility, we transfected hCXCR3-expressing HEK293 cells with another chemokine receptor subtype, hCX3CR1 (also known as fractalkine receptor), and examined migration in response to CXCL10 as well as to the CX3CR1 ligand CX3CL1. After transfection, these HEK293 cells responded significantly to both CXCL10 and CX3CL1 (Figure 2C) with no difference compared with single transfected cells (data not shown).

### hCCX-CKR heteromerizes with hCXCR3 but not with hCCR5

To investigate whether hCCX-CKR might form heteromers with hCXCR3 we performed FRET experiments in HEK293T cells. Co-transfection of hCXCR3-CFP and hCXCR3-Venus revealed high level of FRET efficiency, which was set to 100%, indicating that CXCR3 *per se* shows homomerization



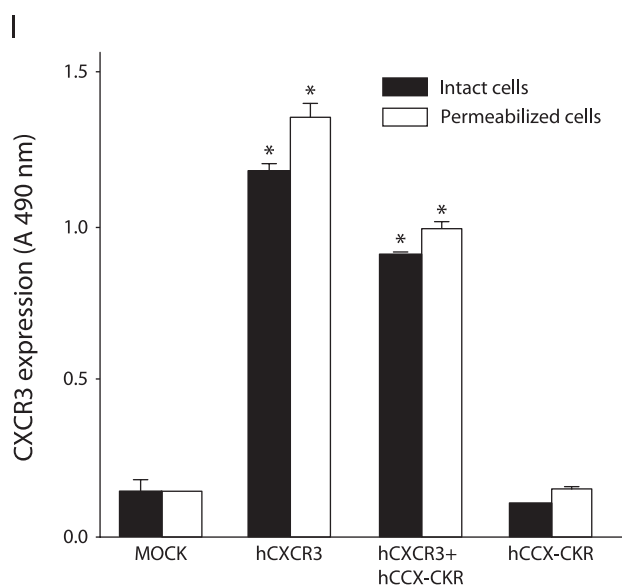
**Figure 1**

Expression of hCCX-CKR does not influence expression of hCXCR3. Immunocytochemical stainings revealed that non-transfected HEK293 cells did not express hCXCR3 (A) nor hCCX-CKR (B), whereas positive labelling was observed in hCXCR3 (D), hCCX-CKR (F), and hCXCR3/hCCX-CKR (H) stably transfected HEK293 cells. Cells stained in the absence of primary antibodies or in presence of an isotype control antibody with irrelevant specificity were devoid of signals (C,E,G). The magnification used in panels A-H was 40X. (I) ELISA experiments showed no influence of hCCX-CKR co-expression on hCXCR3 protein levels when compared with single hCXCR3-expressing cells. No hCXCR3 protein expression was found in mock- or hCCX-CKR-transfected cells. Data represent the means  $\pm$  SEM of three independent experiments. Statistical significance against mock transfected cells was tested by means of multiple comparison ANOVA and Tukey's *post hoc* analysis. Asterisks indicate  $P \leq 0.05$ .

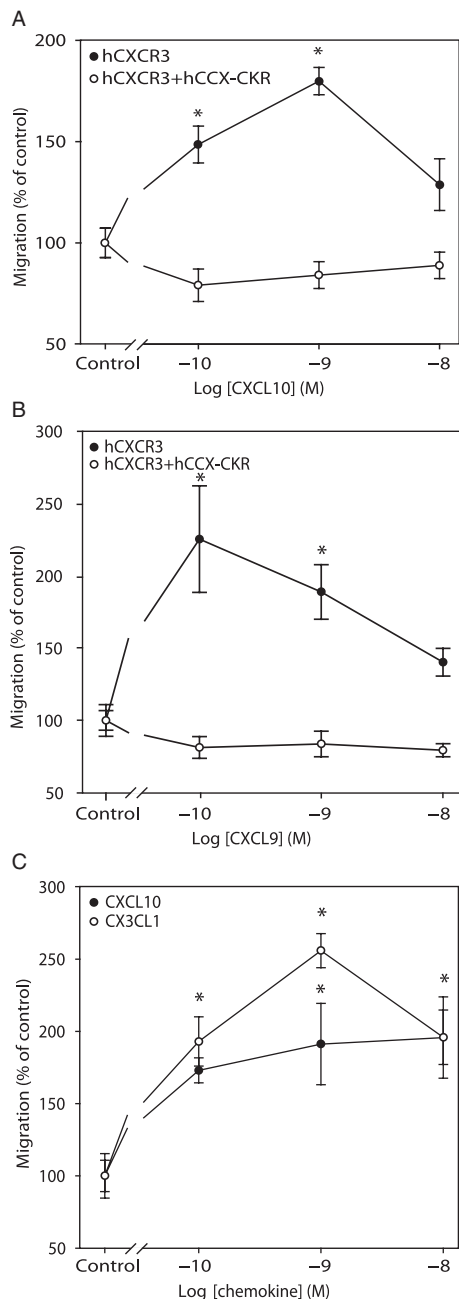
hCCX-CKR was co-expressed with CCR5 or a completely unrelated transmembrane receptor (hGABA<sub>B1</sub>-CFP) no FRET signal was observed ( $0 \pm 3.92\%$ ; Figure 3A,B). These data indicate that hCCX-CKR is able to heteromerize with hCXCR3, but not with CCR5 or GABA<sub>B1</sub>, and thus inhibition of hCXCR3-mediated chemotaxis probably occurs through a direct interaction between both receptors.

### Presence of hCCX-CKR affects binding properties of hCXCR3

Using homologous radioligand displacement assays, we determined the affinities of CXCL10, CXCL11 for hCXCR3 and the affinity of CCL19 for hCCX-CKR in cells expressing the receptors alone or co-expressing CXCR3 and CCX-CKR (Figure 4). Binding affinities of CXCL10 and CXCL11 for hCXCR3 ( $pK_i$  of  $9.7 \pm 0.1$  and  $9.2 \pm 0.1$ , respectively) were not significantly affected by the presence of hCCX-CKR ( $pK_i$  of  $10.0 \pm 0.3$  and  $9.4 \pm 0.1$ , respectively). However, total binding of [<sup>125</sup>I]-CXCL10 was decreased by 47% on co-expression of hCCX-CKR, whereas total binding of [<sup>125</sup>I]-CXCL11 was decreased by 20% (Figure 4A, B). Similarly, the binding affinity of CCL19 for hCCX-CKR ( $pK_i$  of  $8.7 \pm 0.1$ ) was not significantly affected by the presence of hCXCR3 ( $pK_i$  of  $8.9 \pm 0.3$ ). Total [<sup>125</sup>I]-CCL19 binding was decreased by 65% on co-expression of CCX-CKR with CXCR3 (Figure 4C). As negative ligand binding cooperativity has been observed for chemokine receptor heterodimers (Springael *et al.*, 2006; Sohy *et al.*, 2007), we investigated whether this allosteric ligand interactions also occur within the CXCR3-CCX-CKR heterodimer (Figure 5). Next, [<sup>125</sup>I]-CXCL10, [<sup>125</sup>I]-CXCL11 and [<sup>125</sup>I]-CCL19 equilibrium binding was performed in HEK293T cells expressing hCXCR3 alone, hCCX-CKR alone or co-expressing hCXCR3 and hCCX-CKR, in the absence or presence of 100 nM of the unlabeled chemokine receptor ligands CXCL9, CXCL10, CXCL11 (CXCR3) and CCL19, CCL21, CCL25 and CXCL13 (CCX-CKR). Whereas in hCXCR3-expressing cells [<sup>125</sup>I]-CXCL10 binding was displaced only by the CXCR3 chemokines, CXCL9, CXCL10 and CXCL11 (Figure 5A), in cells co-expressing hCXCR3 and hCCX-CKR, negative [<sup>125</sup>I]-CXCL10 binding cooperativity was found for CCL19, CCL21 and CCL25, but not CXCL13 (Figure 5B). CXCL10 did not decrease [<sup>125</sup>I]-CXCL10 binding in cells co-expressing hCXCR3 and hCCX-CKR (Figure 5B). [<sup>125</sup>I]-CXCL11 was displaced by CXCL10 and CXCL11 in cells



(Figure 3A) When the hCCX-CKR-CFP construct was co-expressed with hCXCR3-Venus, a strong FRET signal was observed ( $75 \pm 2.81\%$ ; Figure 3A, indicating heteromerization of hCCX-CKR with hCXCR3. On the contrary, when



**Figure 2**

Expression of CCX-CKR impairs CXCR3-dependent migration. HEK293 expressing stable hCXCR3 migrated towards various concentration of CXCL10 whereas migration of HEK293 expressing hCXCR3+hCCX-CKR was abolished (A). The same migratory behaviour was observed with various concentrations of CXCL9, another CXCR3 ligand (B). HEK293 stably expressing hCXCR3+hCX3CR1 show migratory behaviour towards various concentration of CXCL10 or CX3CL1 (C). Data represent the means  $\pm$  SEM of three independent experiments. Statistical significance was tested by means of multiple comparison ANOVA and Tukey's post hoc analysis. Asterisks indicate  $P \leq 0.05$ .

expressing hCXCR3 (Figure 5C). On co-expression of hCCX-CKR with CXCR3, CCL19 also significantly decreased [ $^{125}$ I]-CXCL11 binding (Figure 5D). Conversely, in cells expressing hCCX-CKR (Figure 5E), the binding of [ $^{125}$ I]-CCL19 was only displaced by CCL19 and CCL21, whereas in cells expressing hCXCR3+hCCX-CKR (Figure 5F) a negative cooperative effect of CXCL11, but not CXCL9 or CXCL10, was observed.

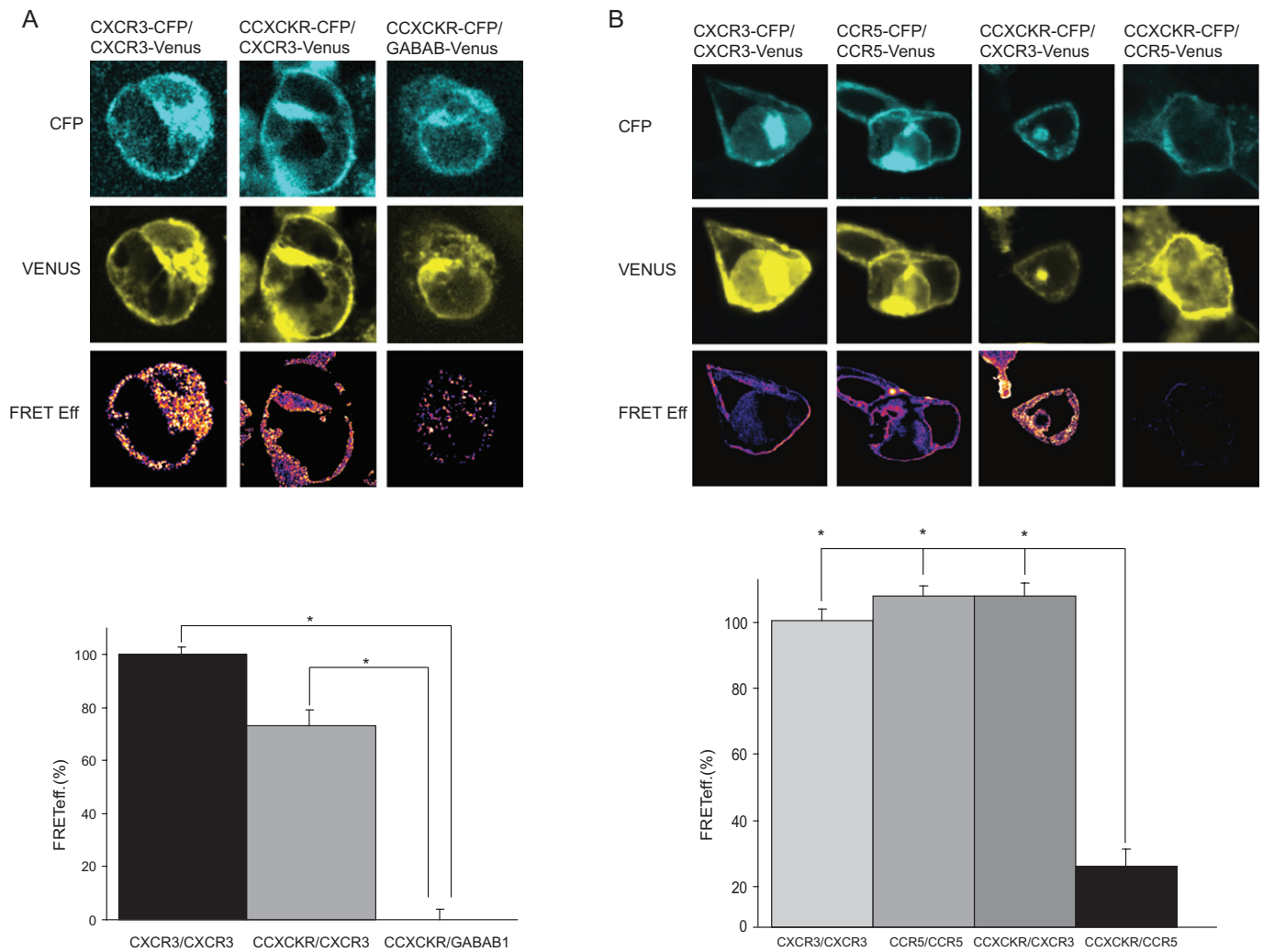
### CXCR3 and CCX-CKR expression in human T cells

Because CXCR3 and CCX-CKR are among the chemokine receptors present in T cells (Loetscher *et al.*, 1996; Gosling *et al.*, 2000; Thomsen *et al.*, 2003), the inhibition of hCXCR3-mediated migration by hCCX-CKR could be of relevance for the migratory behaviour of T cells. Therefore, expression of hCCX-CKR and hCXCR3 mRNA as well as protein was investigated in freshly isolated T cells and T cells activated by phytohemagglutinin (PHA) and IL-2 treatment (Figure 6A). RT-PCR and QPCR analysis showed that both mRNAs were present in freshly isolated T cells (Figure 6B). Whereas PHA/IL-2 treatment did not significantly influence hCXCR3 mRNA expression, a profound down-regulation of hCCX-CKR mRNA expression was observed (Figure 6B). hCCX-CKR and hCXCR3 protein expression was investigated by flow cytometry analysis. It is shown (Figure 6C) that 54.4% of the freshly isolated T cells expressed hCCX-CKR. This percentage dropped to 43.2% positive cells in PHA/IL-2-treated cells (Figure 6C). In contrast a clear increase in expression of hCXCR3 expression was found after PHA/IL-2 treatment, from 18.3 to more than 50.3% CXCR3 positive cells after PHA/IL-2 treatment was observed (Figure 6C). Thus, the treatment with PHA/IL-2 induced a small downregulation of hCCX-CKR and an up-regulation of hCXCR3 expression in human T cells. Similar data were obtained in primary mouse microglia and primary mouse macrophages (Supporting Information Figure S2). Indeed, QPCR analysis indicated the presence of both CCX-CKR and CXCR3 receptors mRNA in these cells. Moreover, treatment with a combination of 50 ng·mL<sup>-1</sup> INF and 100 ng·mL<sup>-1</sup> LPS in the case of primary macrophages and with 100 ng·mL<sup>-1</sup> LPS in the case of microglia provoked a pronounced down-regulation of CCX-CKR mRNA in both cell types (Figure S2A,B). Up-regulation of CXCR3 after activation was observed only in primary microglia (Supporting Information Figure S2A).

### Effect of hCCX-CKR expression on CXCR3 mediated migration in human T cells

Next, chemotaxis of freshly isolated and PHA/IL-2-treated T cells in response to CXCL10 was examined. Whereas freshly isolated T cells did not show migration in response to CXCL10 at all concentrations tested, significant migration was observed in PHA/IL-2-treated T cells, with a peak migratory response at 1 nM CXCL10 (Figure 6D). Thus, similar to HEK293 cells, downregulation of hCCX-CKR was correlated to the migration of human T cells towards CXCR3 ligands. However, since the treatment with PHA/IL-2 caused a robust up-regulation of CXCR3 and does most likely induce various other cellular reactions that could account for the observed change in migratory behavior, we aimed at modifying





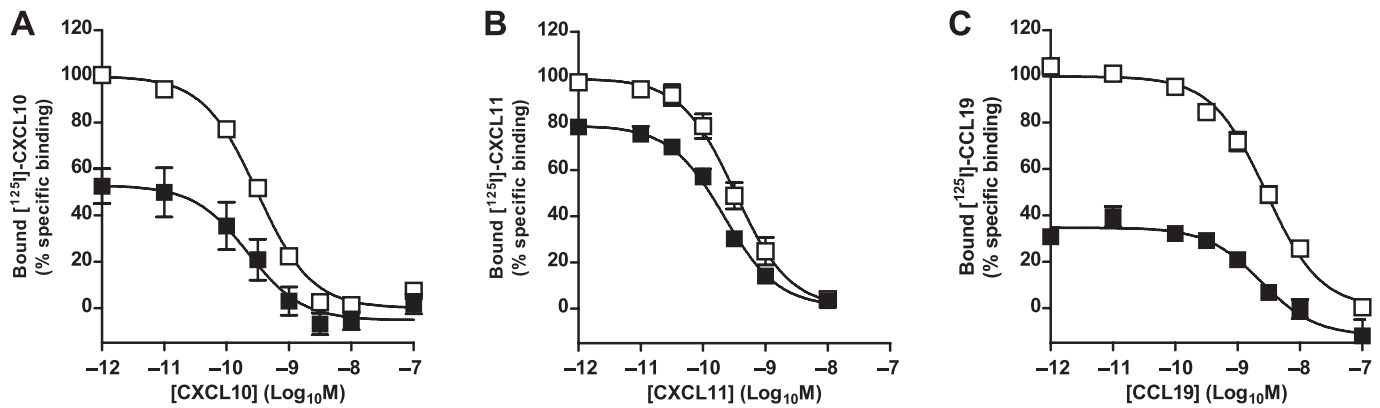
**Figure 3**

hCCX-CR forms heteromers with hCXCR3 but not with hCCR5. (A) HEK293T cells transfected with both hCXCR3-CFP and hCXCR3-Venus constructs showed high FRET efficiency, indicating that both proteins are forming abundant homomeric complexes. When transfected with hCXCR3-CFP and hCCX-CR-Venus, HEK293T also displayed a relatively strong FRET signal, demonstrating that both proteins are interact and form heteromers. HEK293T cells transfected with hGABA<sub>B</sub>R1-CFP and hCCX-CR-Venus failed to show any FRET signal indicating that these receptors do not interact with each other. (B) FRET experiments were performed after co-expression of hCCR5 and hCCX-CR. Strong FRET signals were found for hCXCR3 and hCCR5 homomers. Whereas the heteromer hCXCR3/hCCX-CR showed a strong FRET signal, no signal was observed after co-expression of constructs containing hCCX-CR-venus and hCCR5-CFP. FRET efficiency expressed in % and was scaled on the signal of hCXCR3-CFP and hCXCR3-Venus homomers.

CCX-CR levels in freshly isolated and PHA/IL-2-treated T cells. We therefore transfected freshly isolated T cells with ShRNA for CCX-CR to mimic the down-regulation observed after treatment with PHA/IL-2. This treatment indeed caused a clear down-regulation of hCCX-CR (Figure 7A). Whereas the negative control ShRNA did not affect the chemotactic response of T cells to 1 nM CXCL10, treatment with the ShRNA targeting hCCX-CR clearly enhanced the migration of T cells to CXCL10 (Figure 7B). In addition, hCCX-CR was over-expressed in PHA/IL-2-treated T cells as demonstrated by flow cytometry (Figure 7C). Whereas a significant migration in response to CXCL10 was observed in GFP-transfected cells

(control transfection), no migratory response was observed in PHA/IL-2-treated T cells that had been transfected with hCCX-CR (Figure 7D). Similar data were obtained with primary mouse microglia. Since we observed that treatment with LPS provoked a down-regulation of CCX-CR mRNA and an up-regulation of CXCR3, we hypothesized that activated microglia would migrate more towards CXCR3 ligands. Indeed, we observed a significant increase in cell migration towards both CXCL9 and CXCL10 when microglia were activated with LPS (Supporting Information Figure S2C), showing that the down-regulation of CCX-CR is correlated to an increased chemotactic response.





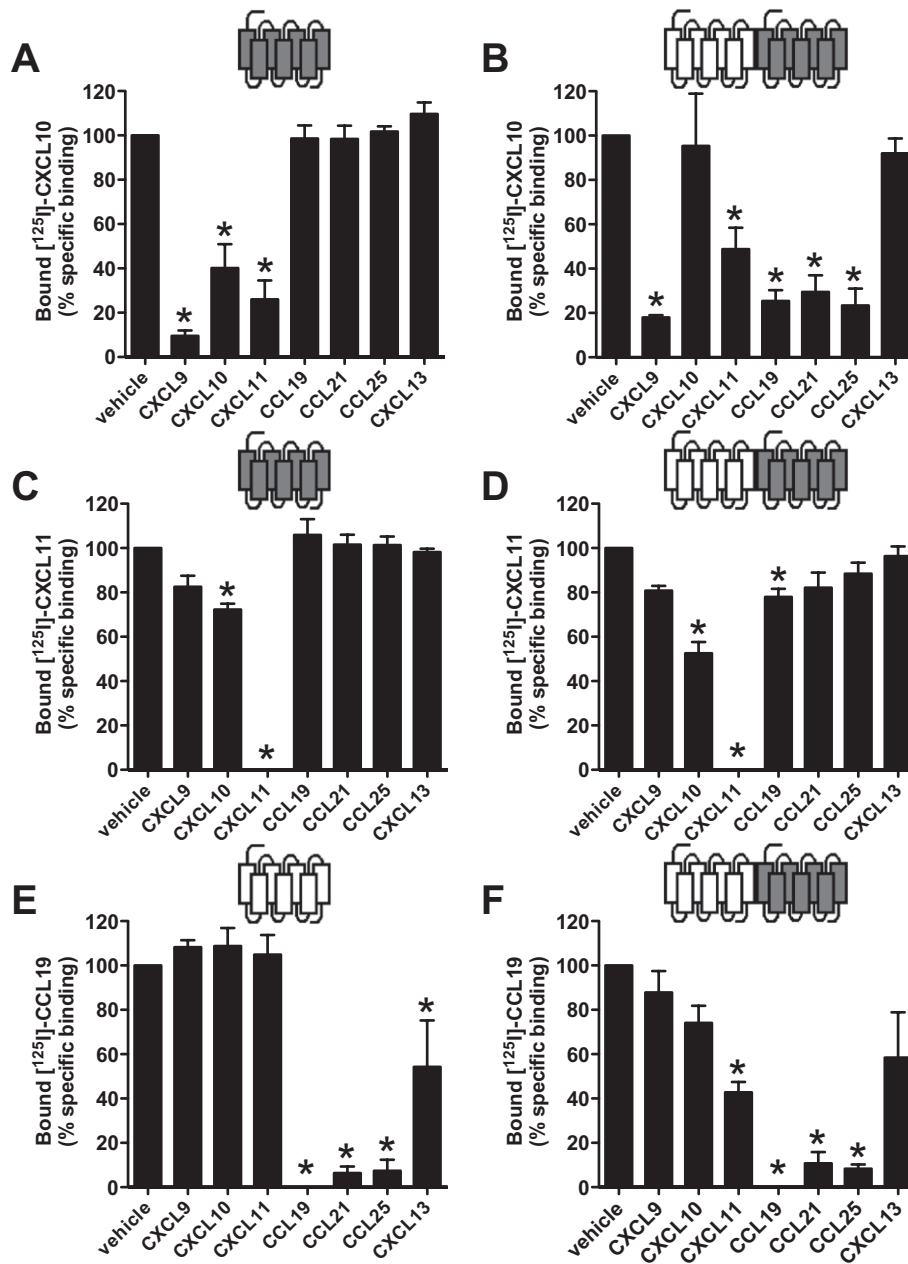
**Figure 4**

hCXCR3 and hCCX-CKR co-expression decreases the number of chemokine binding sites, but not chemokine affinities. Homologous displacement curves were obtained for [<sup>125</sup>I]-CXCL10 (A), [<sup>125</sup>I]-CXCL11 (B) and [<sup>125</sup>I]-CCL19 (C) in cells expressing hCXCR3 alone (A and B, empty symbols), hCCX-CKR alone (C, empty symbols) or cells co-expressing hCXCR3 and hCCX-CKR (A-C, filled symbols). Data were normalized to specific binding in cells expressing CXCR3 or CCX-CKR alone. Data are given as averages ± SEM of specific binding from three independent experiments performed in triplicate.

## Discussion

Atypical chemokine receptors including DARC, D6 have been shown to efficiently internalize their cognate ligands and act as chemokine scavengers (Hansell *et al.*, 2006). Also CCX-CKR binds and scavenges its ligands CCL21, CCL19, CCL25 and CXCL13 (Gosling *et al.*, 2000; Townson and Nibbs, 2002; Comerford *et al.*, 2006). Here, we show that co-expression of hCCX-CKR abolished hCXCR3-mediated migratory responses towards CXCL9 and CXCL10, which is not due to scavenging of hCXCR3 ligands, as CXCL9 and CXCL10 have no affinity for hCCX-CKR (Gosling *et al.*, 2000). It has been demonstrated that chemokines can signal through monomeric and heteromeric receptor complexes (Salanga *et al.*, 2009). Well-known chemokine receptor heteromers are CCR2/CCR5 (Mellado *et al.*, 2001; El-Asmar *et al.*, 2005; Sohy *et al.*, 2009), CXCR1/CXCR2 (Wilson *et al.*, 2005), CXCR4/CCR2 (Percherancier *et al.*, 2005; Sohy *et al.*, 2007; 2009), CXCR4/CXCR7 (Sierro *et al.*, 2007), CXCR4/CCR5 (Contento *et al.*, 2008; Sohy *et al.*, 2009) and DARC/CCR5 (Chakera *et al.*, 2008). In some of these cases, heteromerization had essential implications for agonist-induced chemotaxis and calcium signalling (Sohy *et al.*, 2007; 2009; Chakera *et al.*, 2008). We therefore decided to investigate possible heteromerization of hCCX-CKR and hCXCR3 by FRET. Expressed in HEKT293 cells, hCXCR3 formed homomers and also displayed pronounced heteromerization with hCCX-CKR. In contrast, hCCX-CKR did not heteromerize with hCCR5 or hGABA<sub>B1</sub> receptor, thus confirming the specificity of the FRET assay. Because CCR5-induced chemotaxis is not affected by co-expression of CCX-CKR, this suggests that heteromerization of hCCX-CKR with hCXCR3 plays an essential role in its inhibitory effect on chemotaxis. Heteromerization could induce changes in receptor conformation that possibly would reduce the receptor-ligand binding properties leading to chemotaxis inhibition. Accordingly, the loss of CXCL10 binding sites observed in binding experiments in cell co-expressing hCXCR3 and hCCX-CKR as compared with

cells expressing only hCXCR3, is larger than the loss of CXCL11 binding sites and hCXCR3 cell surface expression as measured by ELISA. Cox *et al.* (2001) have shown that CXCL10 and CXCL11 occupy distinct binding sites at hCXCR3. In contrast to CXCL11 binding, the interaction of CXCL10 with CXCR3 is highly dependent on an active conformation of the receptor. The loss of CXCL10 binding sites in the absence of decreased CXCL10 affinity for hCXCR3 observed in the presence of hCCX-CKR may be explained by a relative increase in the population of CXCR3 in an inactive conformation. Additionally, negative binding cooperativity, induced by ligands both for hCXCR3 and hCCX-CKR was observed. Thus, chemokines for hCCX-CKR suppressed chemokine binding to hCXCR3 and vice versa. In cells co-expressing hCXCR3 and hCCX-CKR, CCL19 could displace both radiolabeled CXCL10 and CXCL11, whereas CCL21 and CCL25 caused significant displacement of [<sup>125</sup>I]-CXCL10 but not [<sup>125</sup>I]-CXCL11. The lack of cooperativity between [<sup>125</sup>I]-CXCL11 and CCL21 or CCL25 may be due to the lower affinity of these two chemokines than that of CCL19 for hCCX-CKR as previously reported by Gosling *et al.* 2000 (i.e. CCL19, CCL21, CCL25, and CXCL13 displaced [<sup>125</sup>I]-CCL19 with IC<sub>50</sub> values of 6, 12, 7 and 140 nM, respectively). The lack of negative binding cooperativity between [<sup>125</sup>I]-CCL19 and CXCL10 may be due to the loss of CXCL10 high affinity binding sites on co-expression of hCCX-CKR with hCXCR3. Additionally, the lack of negative binding cooperativity between hCXCR3 radioligands and CXCL13 is likely due to the moderate affinity of this chemokine for hCCX-CKR. In cells co-expressing hCXCR3 and hCCX-CKR, CXCL10 actually increased [<sup>125</sup>I]-CXCL10 binding, possibly promoting non-specific binding of the radioligand. This effect was also observed in cells that did not express CXCR3 (data not shown). In order to study the potential influence of hCCX-CKR co-expression on hCXCR3 dependent chemotaxis in primary cells experiments with human T cells and mouse microglia and macrophages were performed. Interestingly, in all cells it was observed that the presence of



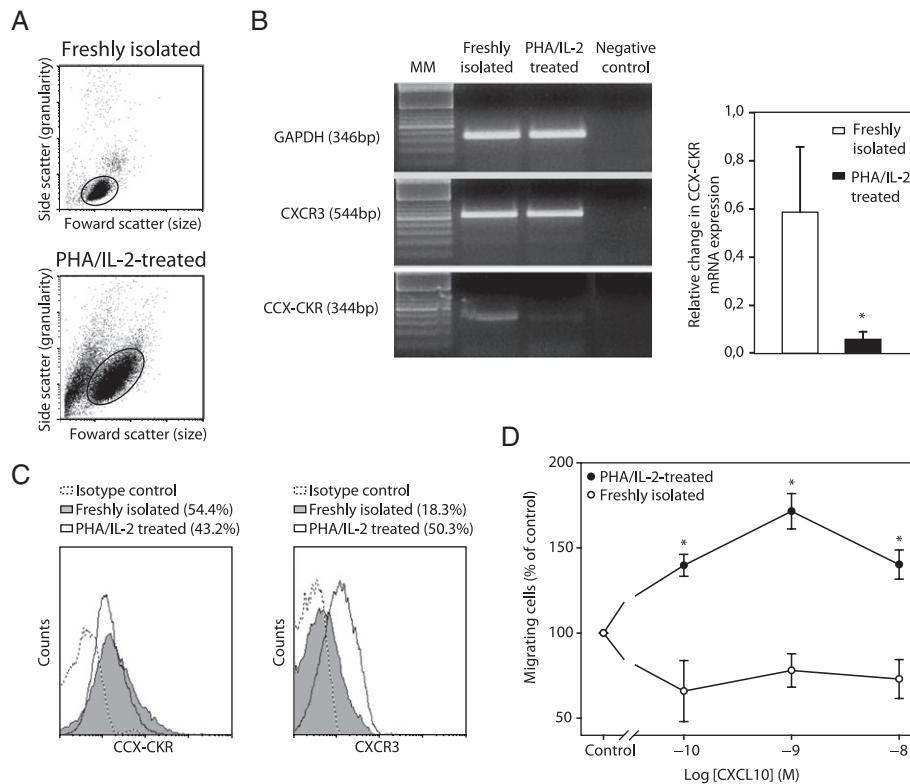
**Figure 5**

Negative ligand binding cooperativity occurs in cells expressing both hCCX-CKR and hCXCR3. [<sup>125</sup>I]-CXCL10 (A-B), [<sup>125</sup>I]-CXCL11 (C-D) and [<sup>125</sup>I]-CCL19 (E-F) equilibrium binding was performed with HEK293T cells expressing hCXCR3 alone (A and C), hCCX-CKR alone (E) or both hCXCR3 and hCCX-CKR (B, D, F), in the absence or presence of unlabeled chemokines (100 nM) as indicated. Data are given as averages ± SEM of normalized specific binding from 2–5 independent experiments performed in triplicate. Statistical significance against vehicle samples was tested by means of multiple comparison ANOVA and Tukey’s post hoc analysis. Asterisks indicate  $P \leq 0.05$ .

hCCX-CKR significantly inhibited the chemotactic response of hCXCR3 ligands. These results thus suggest that co-expression of hCCX-CKR also in a more physiological setting negatively influences the chemotactic response of hCXCR3. In all these cells it was found that an inflammatory activation down-regulated hCCX-CKR expression and thus increased the migratory capacity of these cells to hCXCR3 ligands. It therefore is tempting to speculate that the loss of hCCX-CKR expression is part of the activation programme of immune

cells that enable them to migrate towards sites of inflammation. Interestingly, Gosling *et al.* (2000) described a similar decrease in CCX-CKR mRNA expression for maturing dendritic cells. Whether CCX-CKR might also influence the migration of dendritic cells remains to be established.

Little is yet known about the physiological function of hCCX-CKR. Since no signalling effects of this receptor-like protein have yet been described, hCCX-CKR is currently considered to function as a chemokine scavenger (Comerford



## Figure 6

Expression of hCCX-CKR in freshly isolated and in activated T cells. (A) Freshly isolated T cells and PHA/IL-2-activated T cells were sorted by FACS. (B) RT-PCR analysis show that freshly isolated T cells express both hCXCR3 and hCCX-CKR mRNA. Activated T cells display similar levels of hCXCR3 mRNA as freshly isolated T cells but levels of hCCX-CKR mRNA were considerably lower. \* =  $P < 0.05$ . (C) FACS analysis revealed that 54.4% and 18.3% of freshly isolated T cells expressed hCCX-CKR and hCXCR3, respectively. hCCX-CKR expression dropped to 43.2% in activated T cells whereas a major up-regulation (50.3%) of hCXCR3-expressing-T cells was observed following activation. (D) Chemotaxis experiments show that freshly isolated T cells failed to migrate towards CXCL10 whereas PHA/IL-2-activated T cells demonstrated a prominent migratory behaviour towards the chemokine. \* =  $P < 0.05$  compared with control.

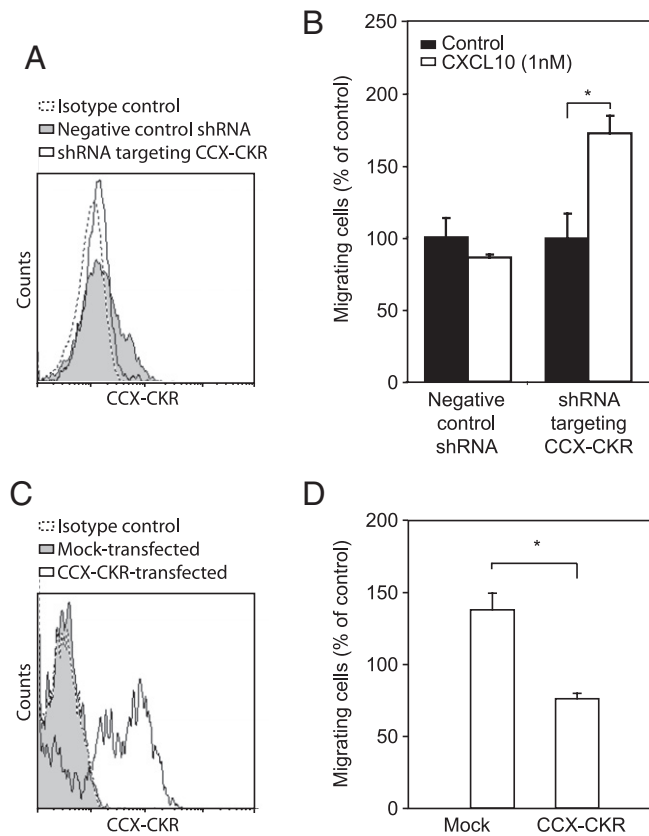
and Nibbs, 2005), similar to the chemokine receptor-like proteins D6 and DARC (Jamieson *et al.*, 2005; Martinez de la Torre *et al.*, 2005). Our current data suggest that hCCX-CKR forms complexes with hCXCR3. This heteromerization might underlie the inhibition of hCXCR3-induced chemotaxis. Negative binding cooperativity upon co-expression of hCXCR3 and hCCX-CKR may also serve as inhibitory mechanism. Similar inhibitory functions have been reported for the atypical CRAM receptor and CCR7 (Catusse *et al.*, 2010). However, contrary to CCX-CKR, which do not display affinity for CXCR3 ligands, the inhibitory effect of CRAM on CCR7 was a combination of ligand competition as well as other unknown factors that might include negative binding cooperativity or heterodimerization. Recently, a possible role of hCCX-CKR as a regulator of growth and metastasis in breast cancer has been reported (Feng *et al.*, 2009). In this study, over-expression of hCCX-CKR inhibited cancer cell proliferation in vitro, and a significant correlation between hCCX-CKR expression and survival rate in breast cancer patients was found. Whether or not heteromerization of hCCX-CKR with other chemokine receptors [as suggested by our chemotaxis experiments (Table 2)] is also involved in inhibition of chemotaxis and proliferation of breast cancer remains to be established.

## Conclusion

Here, we show that hCCX-CKR co-expression has a negative influence on the chemotactic response of the chemokine receptor CXCR3. FRET studies and ligand displacement experiments in hCCX-CKR and hCXCR3 co-expressing cells showed heteromerization and negative binding cooperativity, both of which may influence the migratory capacity of CXCR3. Moreover, we demonstrate that CCX-CKR co-expression also inhibits CXCR3-mediated chemotaxis in human T cells, and mouse myeloid cells. The loss of CCX-CKR expression in these primary cells by activation caused a prominent increase in chemotaxis towards CXCR3 ligands. The inhibition of CXCR3-mediated chemotaxis by CCX-CKR co-expression may therefore be part of the downregulatory programme of non-primed immune cells. Thus CCX-CKR apart from being a scavenger for chemokines may have direct influence on the activity status and chemotactic properties of immune cells.

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### Figure 7

Presence of hCCX-CKR is associated with suppression of chemotaxis towards CXCL10 in T cells (A) Silencing hCCX-CKR using vectors containing DNA encoding for short hairpin RNA targeting hCCX-CKR resulted in a down-regulation of hCCX-CKR expression by freshly isolated T cells as determined by flow cytometry. Data are representative for 4 different human donors. (B) Down-regulating hCCX-CKR rescued chemotaxis towards CXCL10. Data are representative for five different human donors and presented as mean ± SEM. \**P* < 0.05 (Mann-Whitney *U*-test). (C) Flow cytometric analysis showed successful transfection of PHA/IL-2-treated T cells with hCCX-CKR. The data represent one out of five separate experiments with different human donors. (D) Re-expressing hCCX-CKR in PHA/IL-2-treated T cells inhibited CXCR3-mediated chemotaxis towards CXCL10. The data are representative for 6 separate experiments using different human donors and are presented as mean ± SEM. \**P* < 0.05 (Mann-Whitney *U*-test).

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### Conflict of interest

None.

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1 *In situ* hybridization visualizing hCXCR3 and hCCX-CKR mRNA expression in HEK293 cells stably transfected with hCXCR3 + hCCX-CKR.** Stably transfected HEK293 cells were hybridized with sense hCXCR3 (**A**, 200× magnification) or hCCX-CKR (**B**, 200× magnification) probes to determine background staining. Antisense probes for both hCXCR3 (**C**, 200× magnification) and hCCX-CKR (**D**, 200× magnification) revealed perinuclear mRNA accumulation.

**Figure S2 Primary mouse microglia and macrophages behave similarly as human T cells.** Treatment of primary mouse microglia with LPS induced a significant reduction of CCX-CKR mRNA expression, whereas CXCR3 mRNA levels were increased (**A**). mRNA levels of CCXCKR were also downregulated in mouse macrophages when they were treated with LPS and interferon gamma (**B**). Strangely, levels of CXCR3 also were greatly reduced. Chemotaxis experiments with primary mouse microglia show that their migration significantly increase towards ATP, CXCL10 and CXCL9 when treated with LPS (**C**).

**Table S1** Primers used for quantitative real-time PCR.

**Table S2** Threshold cycle number for hCXCR3, hCCX-CKR and the housekeeping gene GAPDH for all HEK293 cell lines.